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INVESTIGATION OF STAPHYLOCOCCAL FIBRINOLYSIN

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16. Abstract

The fibrinolysin activity of 1062 samples of staphylococcal cultures from human, bovine and canine infections was tested. Forty-two of the 790 bovine samples (5.3%), 40 of the 250 human samples (15%), and 11 of the 22 canine samples (50%) were fibrinolysin-positive. With the strongly fibrinolytic staphylococci, the coagulase reaction in the test tube was obscured by the action of fibrinolysin. Therefore, it is concluded that the coagulase slide test should be used in addition to the tube test to detect the coagulase reaction of strongly fibrinolytic streptococci. The method for preparation of "enriched fibrinolysin" is presented, whereby an 80-fold concentration has been achieved.

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INVESTIGATION OF STAPHYLOCOCCAL FIBRINOLYSIN

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Fibrinolysin is considered to belong to the presumed /194* pathogenic factors of Staphylococcus aureus [6, 12]. Fibrinolysin was first identified by means of human staphylococcal cultures, and investigated in connection with coagulase. It was presumed that fibrinolysin and coagulase activity were reactions of a substance (Kleinschmidt [16]; Gratia [11]; Gengou [9]; Reimeter [19], among others). Thus, in the course of an infection, coagulase should first cause coagulation of the plasma, and fibrinolysin should then cause the coagulated plasma to dissolve. This "unitary hypothesis" became untenable as pathogenic staphylococci were isolated, which were indeed coagulase-positive, but demonstrated no fibrinolysin activity. On the other hand, fibrinolysin-positive and coagulase-negative staphylococci have been isolated only seldomly (Christie and Wilson [5]; Kaffka [15], and Smith [21]).

Fibrinolysin has been demonstrated to be abundant in staphylococci of human infections, and, indeed, betweeen 59.8% (Chapman [4]) and 96.4% (Kaffka [15]) of the isolated cultures were fibrinolysin-positive. Of the staphylococcal cultures isolated from cattle, only 5.5% were fibrinolytic (George et al. [10]).

All the staphylococcal cultures isolated from dogs were fibrinolysin-positive (Smith [21]). The isolation of fibrinolysin from staphylococcal bouillon cultures was attempted by

* Numbers in the margin indicate pagination in the foreign text.

Aoi [1] and Fischer [7]. Fibrinolysin was precipitated from the centrifuged remains of the culture with alcohol, acetone, or trichloracetic acid.

The present investigations were carried out with the goal of preparing fibrinolysin in its purest possible form, in order to be able to obtain additional knowledge of the enzyme-like substance in staphylococcal infections.

Material and Methods

Two hundred and fifty staphylococcal cultures from humans[1], 790 from cattle[2,3], and 22 from dogs[4] were used.

The nutrient media were brain-heart-infusion broth (BHIB)[5], and trypticase-soy broth (TBS)[6]. A fibrin-nutrient agar was employed in order to demonstrate fibrinolysin formation. In order to prepare it, 3 g of fibrinogen (bovine fraction I)[7] were dissolved in 100 ml of 0.14 M NaCl. This solution was sterilized by the addition of 3 ml of rabbit plasma citrate by Seitz filtration. The sterile fibrinogen solution was mixed with an equal volume of trypticase-soy-agar solution cooled to 56°C, following autoclaving. The mixture remained in the water bath at 56°C until it attained a milky opacity, and was then poured into prewarmed petri dishes.

Demonstration of the Presence of Fibrinolysin

In order to demonstrate the formation of fibrinolysin, 8-10 staphylococci cultures were spread over fibrin-nutrient agar

[1] Institute for Medicial Hygiene of the University of Giessen.
[2] State Department of Veterinary Investigations, Aulendorf.
[3] Federal Experimental and Research Facility for the Dairy Industry, Kiel.
[4] Institute for Hygiene and Infectious Diseases of Animals, Giessen.
[5] Difco Laboratories, Detroit, Michigan, USA.
[6] Baltimore Biological Laboratory, Baltimore, Maryland, USA
[7] Armour Pharmaceutical Company, Kankakee, Illinois, USA

plates, and fertilized for 24 hours at 37°C. Following fertilization, the fibrinolysin-positive cultures were surrounded by distinct "zones of clarification" (Fig. 1). The fibrinolysin activity of the centrifuged material (16,000 g, 120 min) and filtrate[8] of the bouillon cultures were tested on fibrin agar in small metal cylinders. The fibrin agar consisted of the sterile /195 fibrinogen solution which was mixed with an equal volume of 2% autoclaved agar solution in a water bath at 56°C, and was allowed to become completely opaque at this temperature. The metal cylinders were 0.9 cm tall and had an inner diameter of 0.6 cm, with a wall thickness of 0.1 cm. They were sterilized by dipping in alcohol and finally by passing through a flame, and made contact with the fibrin agar while still warm. The metal cylinders were then covered with a layer of the centrifuged material or filtrates to be investigated. Following an 8-hour fertilization period at 37°C, reaction readings were taken. The diameters of the zones of clarification were takne as an approximate measure of the fibrinolysin activity (Fig. 2).

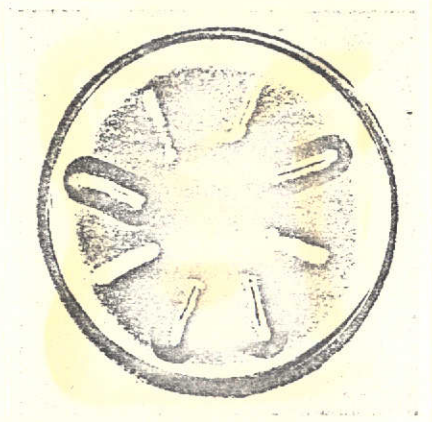

Fig. 1. Demonstration of fibrinolysin on a fibrin-nutrient plate. (The "zones of clarification" (dark regions) about the staphylococcal cultures indicate fibrinolysin activity).

Quantitative Analysis of Fibrinolysin

A fibrinogen plasma coagulase solution was applied to it [14]. A fibrinolysin unit (F.U.) was the smallest quantity of fibrinolysin required to prevent the coagulation of the fibrinogen

[8] Millipore filter (0.65 μm average pore size), firm of Ing. A. Hofmann, Bamberg.

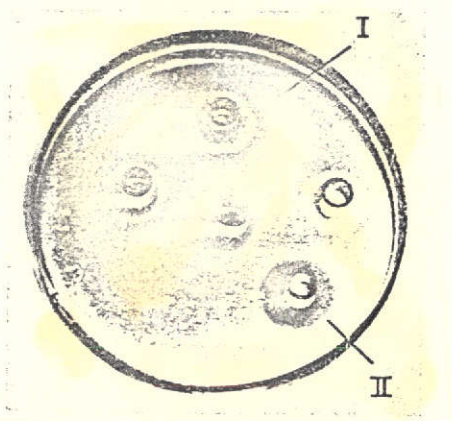

Fig. 2. Demonstration of the presence of fibrinolysin and coagulase from the staphylococcal bouillon culture centrifugate, in metal cylinders on fibrin agar (I = coagulase activity, II = fibrinolysin activity).

solution, following a 3-hour fertilization period at 37°C in the presence of a coagulase unit [3].

Demonstration of the Presence of Coagulase

The tube test was conducted following Fischer [7] and Berger [2] and the slide test, following Williams and Harper [22].

Demonstration of the Fermentation of Mannite

The fermentation of mannite was tested on a mannite bromthymol-blue agar.

Determination of Protein

The presence of protein was determined by the Folin-phenol reagent, following the method of Lowry and co-workers [17].

Concentration of Fibrinolysin

In order to produce fibrinolysin, a culture was sought from a number of strongly fibrinolytic staphylococcal cultures from cattle, which formed neither hemolysins nor coagulase. The staphylococci were fertilized in BHI-bouillon for 48 hours at 37°C, and then centrifuged at approximately 16,000 g for 20 min. In preliminary experiments, fibrinolysin was precipitated from the remains with trichloracetic acid, following Pillet and co-workers [18], with alcohol, following Aoi [1], and with acetone, following /196 Fischer [7]. In addition, enrichment of fibrinolysin with

ammonium sulfate was carried out for 1/4, 1/2 and full saturation. The maximum enrichment of fibrinolysin was attained by means of complete saturation with ammonium sulfate. The precipitate was centrifuged after 12 hours at 4°C, and dissolved in 0.2 M tris-(tris-hydroxymethylaminomethane)-HCl buffer having a pH of 7.0, in approximately 1/100 of the final volume. The fibrinolysin solution was dialyzed against distilled water at 4°C until no further ammonium sulfate was detected by the $BaCl_2$ test. The dialyzed fibrinolysin solution was then precipitated with alcohol at a final concentration of 70% and -20°C. The product was "enriched fibrinolysin." It had an activity of approximately 58 F.U./mg protein. The "enriched fibrinolysin" dissolved in a 0.2 M tris-HCl buffer contained 1024 F.U./ml for all further experiments.

Sephadex Chromatography of the "Enriched Fibrinolysin"

Following filtration, 4 ml of the "enriched fibrinolysin" solution was coated onto a Sephadex column. The chromatography tube had a length of 45 cm and a diameter of 2.5 cm. It was filled with Sephadex G-75[9] in 0.2 M tris-HCl buffer (pH 7.0). The fractions were collected with a fraction accumulator[10], and tested on fibrin agar for their fibrinolysin activity. The protein content of individual fractions was then estimated in the spectrophotometer[11] at 280 mμ.

Attempts to Stimulate Antibodies with Fibrinolysin

"Enriched fibrinolysin served as an antigen for nonhemolytic and coagulase-negative staphylococcal cultures. One milliliter of the antigen solution contained a total of approximately

[9] Sephadex-Pharmacia, Uppsala, Sweden.
[10] Fraction accumulator Radi-Rac, LKB-Productor AB, Stockholm, Sweden.
[11] Zeiss spectrophotometer MPQ II, Oberkocken, Württemberg.

1024 F.U. for a protein content of about 20 mg. Each milliliter of the antigen solution was mixed with 1 ml of 4% sodium-alginal adjuvant[12] with 0.67% calcium gluconate. Rabbits weighing approximately 2.5 kg were injected subcutaneously with this mixture in two different locations. The injections were repeated three times at intervals of 8 days. Serum production occurred 4 days after the final injection.

Proof of Antibody Action Against Fibrinolysin

The antiserum was diluted twofold in 0.14 M NaCl. Each 0.5 ml of the thinned serum was fertilized with 0.5 ml of the "enriched fibrinolysin" for 2 hours at 37°C. A volume of 0.25 ml of this mixture was then combined with the fibrinogen-coagulase solution for the tube test [14]. The tube test was then performed following a 3-hour fertilization at 37°C. For the slide test, another 0.25 ml of the antiserum-fibrinolysin solution was transferred by pipette into a metal cylinder, and fertilized for 8 hours at 37°C.

Immunodiffusion Test

Precipitated antibodies were first detected in the agar gel diffusion test on slides. The slides were covered with a layer of 1% purified agar[13] in 0.14 M NaCl. The antigen was transferred by pipette into punched holes[14] and the antiserum into cut channels[14]. Following 24 hours at room temperature, the slide was examined for precipitate formation.

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[12] "Algivant" -- Colab Laboratories, Inc., Chicago Heights, Ill., USA.
[13] Agar-agar, Riedel de Haen AG, Seelze/Hannover.
[14] LKB-gel Punch-Produkter AB, Stockholm 12, Sweden.

Immunoelectrophoresis

The immunoelectrophoretic separation took place for 1 1/2 hours in the Veronal buffer, pH 8.6, at 250 V, 50 mA, and 4°C. Finally, the laterally cut channels were covered with a layer of antiserum. The immunoelectrophorographs were fertilized for 24 hours at 37°C, and then read. They were dried in a drying cabinet, and then dyed [20] with a 5% amino-black dye (in methanol-glacial acetic acid 9:1).

Results /197

Abundance of Fibrinolysin in Staphylococci of Various Origins

A total of 1062 staphylococci cultures from cattle, humans, and dogs were investigated for their fibrinolysin activity. Of 790 staphylococci from cattle, 42 (5.3%) were fibrinolysin-positive, of 250 from humans, 40 (16%), and of 22 from dogs, 11 (50%) [13].

Fibrinolysin and Coagulase

All fibrinolysin-positive staphylococci were examined with the tube test and slide test for coagulase formation. It was thus determined that several fibrinolysin-positive and mannite-fermented staphylococci were coagulase-negative in the tube test and coagulase-positive in the slide test (Fig. 3). This gave rise to the inference that the coagulase reaction had been obscured in the tube test for several strongly fibrinolysin-active staphylococcal cultures. This is of special importance since the tube test is often the only coagulase test employed for the determination of pathogenicity in routine diagnostic work.

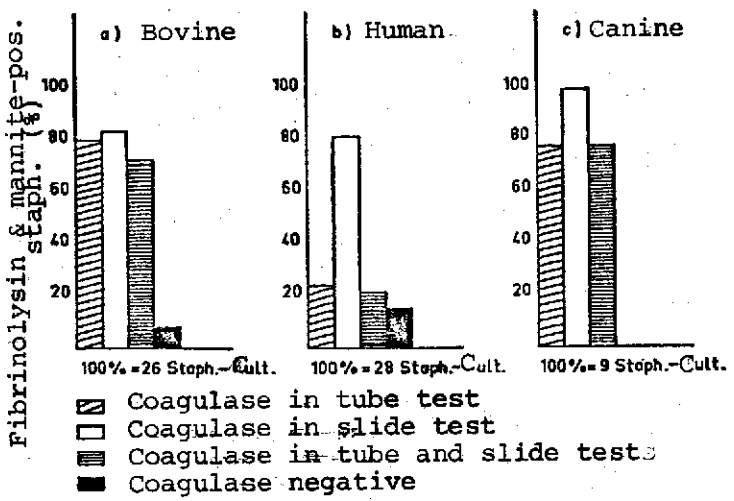

Fig. 3. Coagulase reactions with fibrinolysin-positive and mannite-fermented staphylococci.

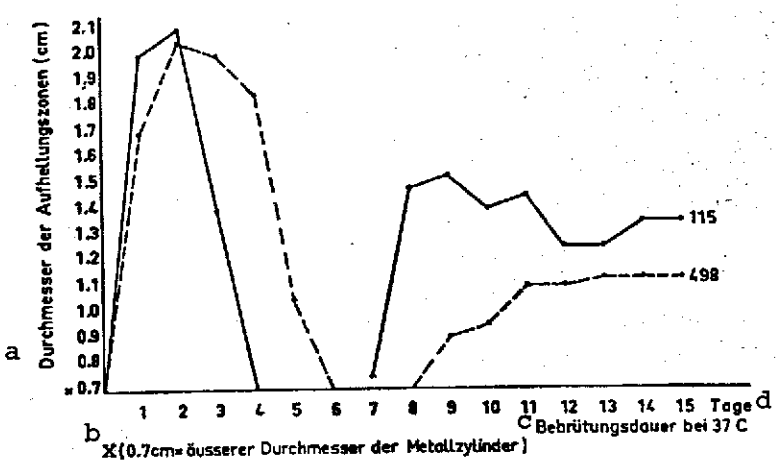

Fig. 4. Fibrinolysin activity of two staphylococcal cultures (115 and 498) during a 15-day fertilization at 37°C.

Key: a. Diameter of the zones of clarification (cm)
b. x(0.7 cm = outer diameter of the metal cylinder)
c. Fertilization period at 37°C
d. Days

Formation of Fibrinolysin

Centrifugates and filtrates of staphylococcal bouillon cultures were investigated daily for their fibrinolysin activity during a 15-day fertilization period. The maximum activity was exhibited following a 2-day fertilization. Upon further fertilization of the cultures, an overall decline in fibrinolysin activity was experienced for a duration of approximately 3 days. After that, a renewed increase in activity was observed, which, however, no longer attained the original maximum (Fig. 4).

Enrichment of Fibrinolysin /198

A coagulase- and hemolytic-negative staphylococcus strain was sought for fibrinolysin production. With it, the biochemical purification of fibrinolysin was simplified from the outset.

Following a 24-hour fertilization of the bouillon culture at 37°C, the remaining bouillon showed a fibrinolysin activity of 0.8 F.U./ /mg of protein. These remains, with fibrinolysin which was precipitated with ammonium sulfate, had an activity of 19.7 F.U./ /mg of protein.

The fibrinolysin which was dissolved in 0.2 M tris-HCl buffer, was precipitated by alcohol of a final concentration of 70% at low temperature. The fibrinolysin purified by alcohol had an activity of 57.5 F.U./mg of protein. In this manner, an approximately 72-fold activity increase was achieved by means of ammonium sulfate and alcohol fractionation.

A further purification of the "enriched fibrinolysin" was achieved with Sephadex. Sephadex G-75 in 0.2 M tris-HCl buffer (pH 7.0) proved to be the best suited of the Sephadex types G-50, -75 and -100. For this reason, Sephadex G-75 was used for the chromatographic purification of fibrinolysin (Fig. 5).

TABLE 1. ENRICHMENT OF FIBRINOLYSIN

Enrichment stages	Specific fibrinolysin activity
Centrifugate remains of staph. bouillon cultures	0.8 F.U./mg protein
Precipitation with $(NH_4)_2SO_4$	19.7 F.U./mg protein
Precipitation with C_2H_5OH	57.5 F.U./mg protein
Sephadex chromatography	65.0 F.U./mg protein
Total enrichment	approximately 80-fold

The chromatographically separated fibrinolysin had a specific /199 activity of approximately 65 F.U./mg of protein. An approximately 80-fold increase in fibrinolysin activity could be achieved with respect to the fibrinolysin activity of the bouillon culture centrifugate (Table 1). The further purification of fibrinolysin could also be demonstrated immunoelectrophoretically (Fig. 6a).

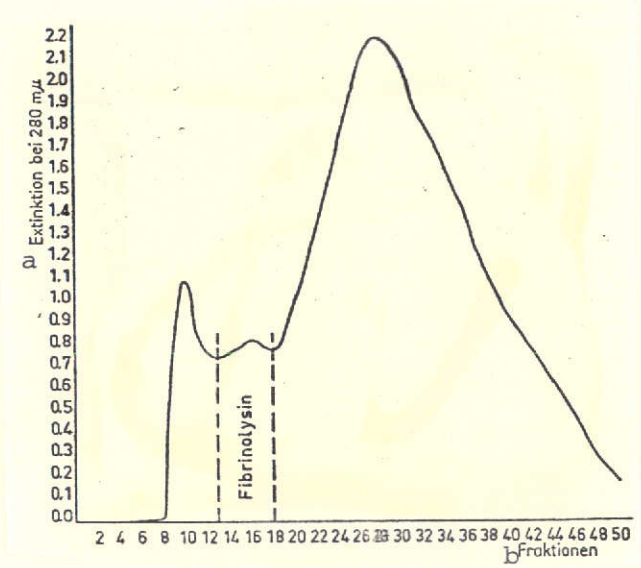

Fig. 5. Chromatography of "enriched fibrinolysin" with Sephadex G-75 (Zeiss spectrophotometer, 280 mμ). The fractions inside the dashed region are fibrinolysin activity.

Key: a. Extinction at 280 mμ
b. Fractions

Stimulation of Antibodies /200 by Means of Fibrinolysin

The serum from rabbits, vaccinated with "enriched fibrinolysin," were tested 4 days following the fourth injection for their antifibrinolytic effect. A significant inhibition of fibrinolysin activity was thereby observed in the tube test. This inhibition was not, however, unambiguous in the slide test.

Indentification of Precipitation Lines in the Immunoelectrophorogram

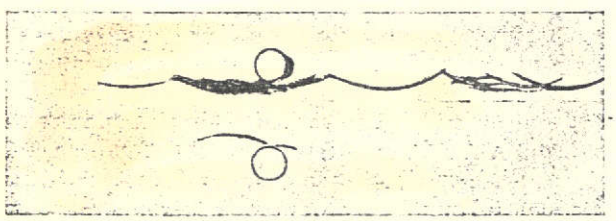

Fig. 6a. Immunoelectrophoresis of the "enriched" (above) and the chromatographically purified fibrinolysin (below) with the antiserum for "enriched fibrinolysin".

The immunoelectrophorograph was used as a purity standard for the individual stages of concentration. A parallel electrophorograph was coated with the fibrin agar immediately following the 1 1/2 hour electrophoresis, and the whole thing was fertilized for 24 hours at 37°C in a humid chamber. The zones of clarification which formed in the fibrin agar indicate the location of the fibrinolysin fractions in the electrophorograph (Fig. 6b). A different diffusion rate in an electric field was determined between the "enriched fibrinolysin"

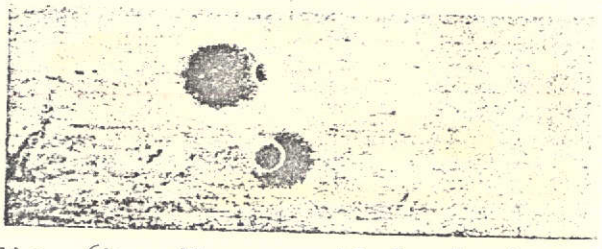

Fig. 6b. The parallel electrophorograph, covered with fibrin agar, following 12 hours of fertilization at 37°C. The "zones of clarification" indicate the presence of fibrinolysin fractions.

and the chromatically separated fibrinolysin, by means of the positions of the zones of clarification.

Discussion

Various assertions have been made concerning the presence of fibrinolysin in the staphylococci of humans and animals [1, 5, 6, 15]. In individual experiments, 42 of 790 (5.3%) staphylococcal cultures from cattle, 40 of 250 (16%) from humans, and 11 of 22 (50%) from dogs were found to be fibrinolysin-positive. The increment of fibrinolysin-positive staphylococcal cultures from cattle is thus in agreement with the results of George et al., [10].

The enrichment of fibrinolysin has been investigated by a number of authors [1, 7, 18] by precipitation of the bouillon culture remains with alcohol or trichloracetic acid. Using this procedure, the separation of coagulase and fibrinolysin is extremely difficult. This can be avoided from the outset by using a coagulase- and hemolytic-negative, strongly fibrinolytic staphylococcal culture. An approximately 72-fold increase in fibrinolysin activity can be achieved by precipitating the bouillon culture remains with ammonium sulfate and finally with alcohol. The purification could also be performed immunoelectrophoretically.

The variable diffusion rates of fibrinolysin in its "enriched" and chromatically prepared forms can perhaps be traced to the fact that the fibrinolysin may have been closely bound to other substances before the chromatographic separation, which would influence the total charge.

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